Introducing new effector function to IgG via protein engineering of an IgA-IgG hybrid antibody

Presented by Nishant Mehta

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1. Introduction

Biotechnology is the utilization of biological organisms, processes, and systems for the generation of useful products. A significant component of the biotechnology industry is comprised of pharmaceutical companies that make and market therapeutic drugs. In recent years pharmaceutical companies have shifted significantly from the creation of chemically synthesized small molecule drugs to new biologic drugs produced in living hosts. In 2009, intellectual property law firm Withers & Rogers released a report showing that patent filings for biologics accounted for 60% of the total patent filings from the top 10 pharmaceutical companies.[1] Additionally, the biopharma industry consultancy group KMR released a report showing that 25% of biologics in Phase II reached the market compared to 10% of small molecules between 2006 and 2010.[2] Amongst this classification of biologics are antibody drugs which hold a market share of more than 10 billion dollars.[3]

Because of their interaction with immune effector cells, antibodies have a plethora of current and potential uses. In terms of therapeutic application, antibodies can be used to treat viruses, autoimmune diseases, and even various cancers. Utilization of the human body's own immune cells is a major advantage for antibody-based therapeutics. The potential advantages and diverse applications of antibody based drugs have contributed to the pharmaceutical shift towards biologics.

1.1 Antibody structure

The basic structure of an antibody is comprised of two identical heavy chains and two identical light chains assembled as a tetramer similar in shape to the letter Y, as shown in figure 1. Each heavy chain has four protein domains with interchain

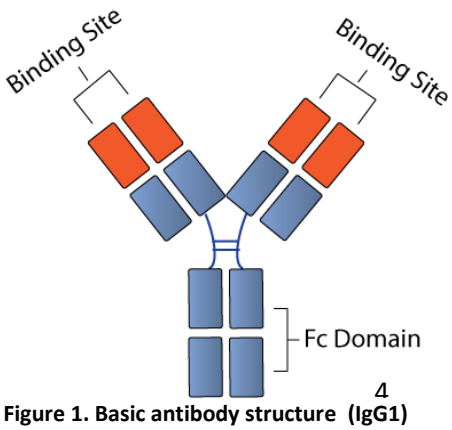


Figure 1. Basic antibody structure (IgG1) showing the different protein domains.

disulfide linkages in the hinge region. Light chains have two domains which are often disulfide linked to the CH1 domain of the heavy chain. The antigen binding sites are located at the intersection tip between the light chain and the heavy chain. Binding specificity is determined by regions of high variability termed complementary determining regions (CDRs). Genetic recombination and error prone mechanisms within the bone marrow can produce up to 10^{11} unique variable region combinations.[4] Finally, the Fc domain is the heavy chain protrusion at the bottom. The Fc domain is responsible for binding to Fc receptors on the innate effector cells. Antibody-antigen binding facilitated by protein variable regions stimulates an immune response which is mediated by the binding between Fc domains and immune effector cells.

1.2 The immune response

Rather than grouping together in a one-to-one pair, multiple antibodies bind to multiple antigens in a higher level complex. This process is known as agglutination (Figure 2). The formation of this complex helps facilitate efficient ADCC, or antibody-dependent cell mediated cytotoxicity, by assembling microbes together to increase effector cell accessibility. Multiple interactions between antibody and antigen introduce an avidity effect resulting in strong attachment. When antibody-antigen binding is significantly high, the antibody Fc domains cross link receptors on effector cell surfaces to overcome the threshold required for activation. Methods of antigen destruction vary from phagocytosis via macrophages or neutrophils to perforin and granzyme mediated induction of apoptosis via natural killer cells.[5] Antibody function is mediated through differential binding patterns to various Fc receptors. IgG acts through Fc gamma receptors and IgA acts through Fc alpha receptors.

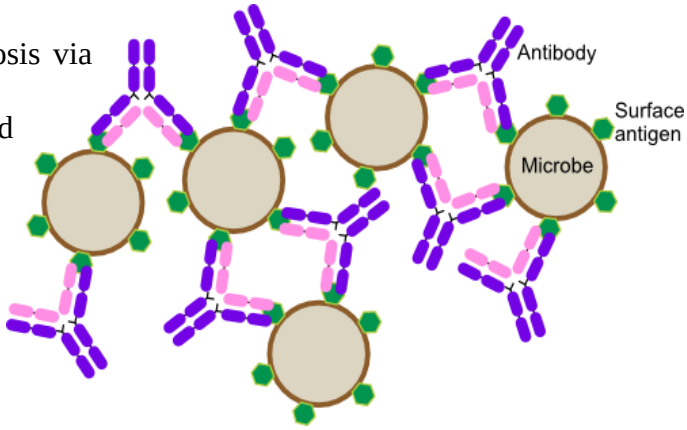


Figure 2. Antibody agglutination. Antigen binding sites on each antibody pair up with surface antigens present on microbes.[3]

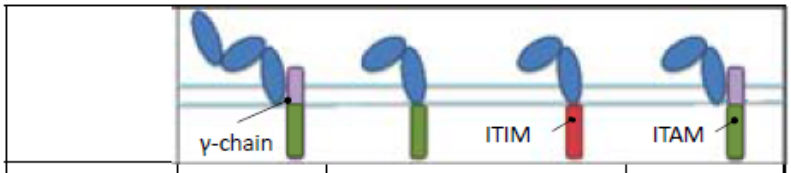
1.3 Antibody classes and their receptors

Five different classes of antibodies are naturally synthesized by the human body. Of these, IgG and IgA have the highest concentration in human serum and have shown therapeutic potential.[6]

1.3.1 IgG and Fcγ Receptors

The most abundant class in serum is immunoglobulin G (IgG). IgG contains two variable binding domains and one effector cell binding domain (Fc domain). All FDA approved antibody drugs thus far have been made with an IgG1 backbone as this isotype has the highest binding to certain important inflammatory receptors.[7]

The Fc gamma receptor class is made up of five receptors: FcγRI (CD64), FcγRIIA (CD32), FcγRIIB (CD32), FcγRIIIA (CD16a), and FcγRIIIB (CD16b). Expression profiles of each Fcγ receptor are shown in Figure 3.[8] FcγRI has been shown to mediate ADCC and participate in endocytosis leading to phagocytotic function of effector cells.[9] FcγRIIA is a commonly regarded killing receptor that is known to stimulate phagocytosis and neutrophil degranulation.[10,11] FcγRIIB, on the other hand, is the only Fc gamma receptor that has an inhibitory function. When a pathogen-bound IgG binds to FcγRIIB, granted the activation threshold is reached, the cytotoxic process comes to a halt. Activating FcγRs in the proximity of bound FcγRIIB are also adversely affected.[12] Finally, FcγRIIIA is an activating receptor that is known to facilitate phagocytosis, induce ADCC through natural killer cells, and stimulate the release of inflammatory mediators.[13,14]



Class	FcγRI	FcγRII		FcγRIII
Subclass	FcγRI	FcγRIIa	FcγRIIb	FcγRIIIa
Expression	Macrophages Monocytes DCs Eosinophils Neutrophils	Macrophages Platelets Monocytes DCs Neutrophils	Macrophages Monocytes DCs Eosinophils Neutrophils Mast cells B cells	Macrophages Monocytes DCs Mast cells NK cells

Figure 3. FcγR expression patterns. Green=Activating, Red=Inhibiting.

1.3.2 IgA and FcαRI

Immunoglobulin class A (IgA) is the most common type of antibody in the mucosa and the second most abundant in the serum. IgA antibodies are represented in higher serum concentrations in humans than in the sera of other animal species. In serum, IgA is predominantly monomeric. However IgA present in secretions, i.e. bile, saliva, nonvascular fluids, is usually dimeric. Human IgA can be further divided in to two different subclasses: IgA1 and IgA2. The structural difference between these subclasses is the absence of a thirteen-amino acid sequence in the hinge region of IgA2.[15] Understanding the interaction between IgA and its Fc receptor is important for recognizing the role that an IgA/IgG hybrid antibody would play as a therapeutic.

Most of the investigated IgA therapeutic effects are generated through interaction with FcαR1 (CD89). This Fc receptor is expressed on cells of the myeloid lineage including macrophages and neutrophils. Human FcαRI binds to a region of IgA between the CH2 and CH3 domains in the Fc. The IgA-FcαRI complex activates a variety of antigen killing reactions such as respiratory burst, phagocytosis, and cytokine production. Unfortunately, the circulation half-life of IgA is significantly less than that of IgG at 5.9 days for serum IgA1 and 4.5 days for serum IgA2 (IgG can last up to 23 days depending on isotype).[16] Despite this drawback, IgA has therapeutic potential due to its strong interaction with immune effector cells, specifically neutrophils, the most common form of white blood cell in mammals.[6]

Neutrophils form an essential part of the innate immune system by responding quickly to sites of inflammation. These cells are phagocytes that are able to ingest and destroy targets that are coated with opsonins, or molecules that present bound antigens. As part of the cell killing mechanism, neutrophils are also able to release oxygen radicals, enzymes, and antimicrobial

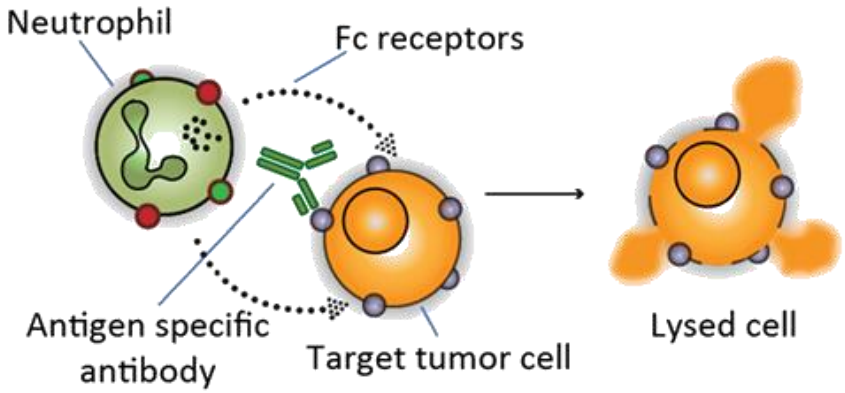


Figure 4: Neutrophil mechanism for target cell killing

peptides to cause target destruction (Figure 4). Most of these proteins are stored in granules which are released in a process called degranulation.[17]

1.3.3 IgA vs. IgG

The advantages of using IgA as a therapeutic over IgG are related directly to neutrophil recruitment and activation. It has been demonstrated that FcαRI is the most potent neutrophil Fc receptor to induce antibody-dependent tumor cell killing.[18] In fact, in a side by side comparison, the degree of neutrophil-initiated colon carcinoma cell killing was higher in the presence of IgA1 mAbs compared to an IgG1 counterpart.[19] Additionally, IgA does not bind FcγRIIb, the gamma class inhibitory receptor. Since FcαRI is the only Fcα receptor, no inhibitory receptor analog exists for the α class. Its ability to potentiate the effect of inflammatory neutrophils through CD89 and its circumvention of inhibitory receptor binding makes IgA a powerful potential therapeutic. However, this potential for potency can only be realized through the precise design and engineering of antibody mutants.

1.4 Antibody Engineering

More often than not, protein therapeutics are made from antibodies. Antibody variable regions are often discovered through a rational trial and error process that involves the injection of a specific antigen into a mouse. The murine immune system will detect the foreign material

and create a pool of polyclonal antibodies against it. Recently, Sanofi and partner Regeneron have engineered mice that have fully human immune repertoires that can produce entirely human antibodies.[20] These antibodies can be expressed and selected for certain binding characteristics using hybridoma cell lines, or B lymphocytes fused with immortalized myeloma cell lines.[21] Proliferating B cells generated from the same lineage can produce monoclonal antibodies. Monoclonal antibodies (mAbs) are used therapeutically because every clone binds to the target antigen in the same manner; in contrast, the heterogeneity of polyclonal antibodies can be risky for a patient. With the development of modern cloning techniques, researchers are now able to isolate B cells and clone select sequences in to expression vectors that can be screened using techniques such as phage display or periplasmic expression.[22,23] In particular, engineering a hybrid antibody consisting of two different classes requires specific modifications of the Fc domain.

There are two predominate methods for engineering better therapeutic antibodies: variable domain manipulation and Fc manipulation. The variable domain of an antibody targets the antigen while the Fc domain binds to receptors on various immune cells to affect potent cellular responses. Increasing binding efficacy between Fc domain and Fc receptor can augment drug potency. In the treatment of cancers, high avidity antibody-antigen complexes ligate several Fc receptors together on a responding immune cell to initiate intracellular signaling pathways that ultimately trigger the release of cytotoxic components and cause the destruction of the tumor cell. By improving the binding to the Fc receptor, one can recruit more receptors and potentially generate stronger immune responses. Moreover, since toxicity is almost entirely dependent on drug dose concentration, a more effective drug that requires lower dosage is safer for the patient.

2. Creation of an IgA/IgG Hybrid Antibody

The ability to bind two different classes of Fc receptors is a desirable trait for an antibody based therapeutic. The IgG and IgA classes are the first and second most prevalent antibody isotypes in the human body.[24] Most antibody drugs use IgG as a backbone because it is the most effective agent of ADCC due to its natural prevalence.[25] However, combining regions from different classes to yield an antibody with dual binding has significant potential. IgA was the selected as the desired second class because of the potency of the FcαRI receptor. Its high expression on the surface of neutrophils makes FcαRI a powerful Fc receptor for the purposes of antibody based tumor cell killing.

2.1 Previous Hybrid Fc Engineering Work

Previous work from other labs on the creation of hybrid IgG/A focused on the substitution of entire heavy chain constant domains and the fusion of additional protein domains to IgG to investigate FcαRI binding. The first hybrid protein was a domain swap variant in which the entire IgG CH3 (constant region 3 of the heavy chain) was replaced with the IgA CH3 analog. However, this variant was found to have no binding to FcαRI.[26] More recently, a hybrid variant was constructed by attaching the IgA CH3 domain to the C-terminus of the IgG heavy chain.[27] The new variant was able to retain FcγRI binding and could dimerize via J-chain linking of the IgA CH3 peptide appendages. However, the variant showed lower serum persistence due to the higher avidity interactions with FcRn. FcRn is an Fc receptor responsible for the recycling of immunoglobulin to prevent lysosomal degradation. Antibodies bind to FcRn and are recycled to the cell surface.[28] The extra heavy chain domain in the γγγα variant increased the avidity interactions with FcRn which prevented endosomal release on the cell surface. Lower half-life due to ineffective FcRn recycling prevents this mutant from being an effective therapeutic.[27]

These past experiments with hybrid variants show the potential for IgG/A hybrid antibodies but also reveal the problems that must be overcome in the protein engineering process.

2.2 Project Rationale

Engaging both FcγRI and FcαRI using bispecific antibody fusions for in vitro cell killing has been successful in the past. An ADCC assay was done in the presence of two antibody fusions, one that bound a surface antigen as well as FcγRI and a second that bound the same surface antigen as well as FcαRI. Together, this combination destroyed tumor cells to a higher degree than either antibody alone.[24] However, injecting a patient with two different types of antibodies is not ideal. First, matching glycan heterogeneity profiles for two different antibodies in a single drug would be very time consuming and expensive. The FDA would be hesitant to approve a drug with such high batch to batch inconsistency. Second, this potential drug would bind to the inhibitory FcγRIIb receptor thereby decreasing potency. Our approach builds on this finding and avoids these safety problems by making one single antibody backbone. Furthermore, cancer patients in particular are often immunocomprimised; thus, there is a pressing need for treatments that recruit more abundant immune cell populations. FcαRI is highly expressed on neutrophils which are well represented in serum (~70% of all effector cells). Our goal is to create a variant that can use these cells to induce the desired immune response

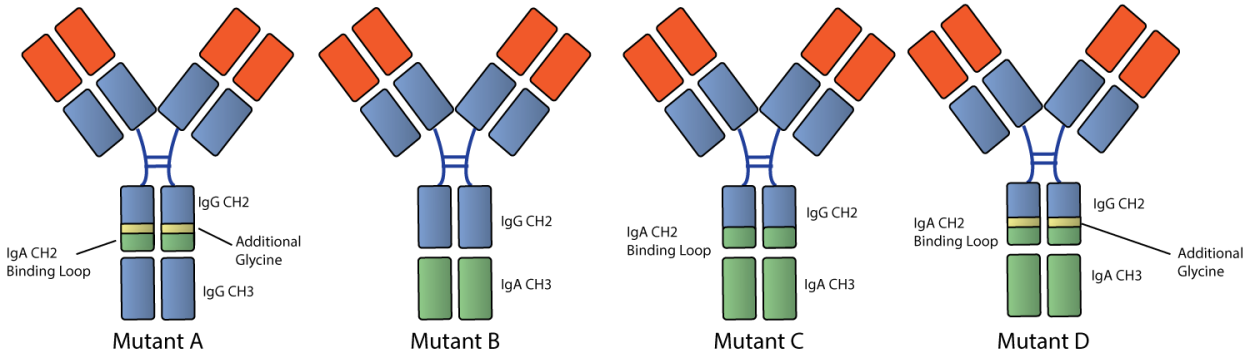
To achieve hybrid binding, we decided to adopt an approach that incorporates the domain swap ideology with other rational design techniques. First, we examined the crystal structure of the IgA-FcαRI complex using protein modeling software called PyMOL. Different residues were tested for binding importance through a process called alanine scanning. We isolated a ‘protein loop’ of the CH2 region of IgA that seemed to be very important for binding to FcαRI. Additionally, there were many residues in the CH3 domain that could be considered binding

hotspots. However, changing residues haphazardly can result in undesired changes in tertiary and quaternary structure. As a result, the protein may have trouble folding and may not express well in bacterial or mammalian cells. To circumvent this issue, we decided to graft the entire CH3 domain from IgA in to IgG. Our hope was that inserting an entire domain from a protein that is known to fold and express well rather than changing individual residues would result in higher binding retention. Also, substituting large domains would preserve recognizable amino acid sequences and could result in lower immunogenicity. The upstream CH2 binding loop from IgA was also included in our first round of mutant designs. Our plan was to make three mutants using this form of rational design and then optimize FcaRI binding further using directed evolution approaches.

3. Engineering Methods

3.1 IgA/G Variant Design

Four different combinations of the IgA CH3 domain and IgA CH2 binding loop were grafted in to IgG. Specifically, mutant A replaces IgG1 residues 245-258 (KGQPR….LSPGK) with IgA residues 251-263 (PALEDLLLGSEAN) including an additional glycine after IgA residue. Mutant B exchanges IgG1 residues 340-447 (KGQPR….LSPGK) with IgA1 residues 340-450 (SGNTF…KTIDR). Mutant C replaces IgG1 residues 340-447 (KGQPR….LSPGK) and residues 245-258 (PKPKDTLMISRTPE) with IgA1 residues 340-450 (SGNTF…KTIDR) and 251-263 (PALEDLLLGSEAN) respectively. Similarly, mutant D includes the two swaps from mutant C but also adds a glycine after IgA residue 263 (after the CH2 binding loop). The additional glycine in mutants A and D was added to match the loop length of IgG and also to add flexibility for folding. Figure 5 shows the four initial binding mutants. (Note: Mutant A was constructed after characterization of Mutants B,C,D).



3.2 Construction and Expression of IgA/G Variants

Mutants A-D were made via overlap extension PCR and cloned into the vector pMaz-FcγRI-His. The dpnI digested product was then transformed in to JUDE-1 electrocompetent cells. Plates were grown overni ght and colonies picked for subculture in to bacterial culture

flasks. Plasmid DNA was prepared by miniprep (Qiagen) for transfection. 293Fectin Transfection Reagent (Invitrogen) was used to transfect HEK293F (Invitrogen) cells cultured in GIBCO FreeStyle™ 293 Expression Medium (Invitrogen) in accordance with the manufacturer's instructions. 5-6 days after transfection the cell suspension was centrifuged at 2000 rpm for 10 mins to recover the supernatant fraction. The protein supernatant was then purified using an Ni-NTA purification system to isolate the proteins via His-tag affinity. 25x PBS was added to the protein supernatant to a final concentration of 1x in addition to 10mM imidazole. The solution was passed through Ni-NTA affinity columns for protein binding. Bound protein was washed with 20 mM imidazole in PBS and eluted with 250 mM imidazole in PBS directly into Amicon 10kDA spin columns for buffer exchange and concentration.

3.3 ELISA Testing

Ni-NTA ELISA plates (Qiagen) were coated with 4ug/mL of each of the mutant Fc domains (Sino Biological) in 1x PBS containing 0.05% Tween (PBST) and washed three times in PBST at pH 7.4. To the first well 66.66 uL of 10 ug/mL of each of the test Fc receptors (FcαRI-GST, FcγRI-GST, FcγRIIa-R131-GST, FcγRIIb-GST or FcγRIIIa-F158-GST) dissolved in PBS with 2% milk (PBSM) was added followed by a 1:4 serial dilution down the plate. After 1 hour of incubation at room temperature, the plates were washed and 50 uL PBSM with goat anti-GST HRP (GE Healthcare) 1:5000 was added. Following 1 hour incubation, the plates were washed 3x with PBST and developed with 50 uL TMB substrate per well (Thermo Scientific). To neutralize, 50 uL of 1 M H_2SO_4 was added per well and the absorbance at 450 nm was recorded. This ELISA assay protocol was repeated later with mutant A to examine FcγRIIIA binding.

3.4 Alanine Mutagenesis

Based on crystal structure data and Rosetta modeling, 11 sites in the IgA Fc domain that were identified or suspected to interact with FcαRI were selected for alanine scanning (Figure 6). Eleven Fc mutants, each with a single alanine substitution, were made using overlap extension PCR. After the mutants were cloned and purified, ELISA analysis was performed to compare the binding to wild type (WT) IgA. Costar 3590 96-well plates were coated with our 11 mutants. Next, FcαRI-GST was added at 20 µg/ml and binding was detected with anti-GST HRP. Binding efficacy was compared to IgA and IgG as positive and negative controls, respectively. Additionally, 2 µg of each mutant protein was loaded in to a 4-20% SDS-Agarose Protein Gel (Invitrogen) and run at 110 W for 30 mins.

3.5 Surface Plasmon Resonance (SPR) Analysis

Surface plasmon resonance analysis was carried out using a BIAcore 3000 instrument to determine binding kinetics (GE Healthcare). Five nanomoles of the mutant of interest (mutant D) and the two controls were immobilized on CM5 sensor chips by amine coupling as recommended by the manufacturer. The dissociation experiments were performed in HBS-EP buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3.4 mM EDTA, and 0.005% P20 surfactant) (GE Healthcare). Dimeric forms of FcαRI-GST, FcγRIIb-GST, FcγRIIa(H)-GST, and monomeric FcγRI were injected in duplicate at a rate of 30 µl/min for 60 seconds. A dissociation time of 5 minutes was set to examine the protein-ligand binding kinetics. After each injection, the chip was regenerated to remove both the ligand and the analyte for further machine runs. For the dimeric receptors, the chip was regenerated by sequential injection of 50 mM glycine (pH 4.0), 50 mM glycine (pH 9.5), and 3 M NaCl for 2 min. each. For the monomeric FcγRI receptor, the chip was regenerated by injecting 10 mM glycine at pH 3.0 between samples. Equilibrium dissociation

constants (KD) for monovalent receptor binding were calculated by fitting a 2:1 bivalent analyte model to the data using BIA evaluation 3.2 software (GE Healthcare).

3.6 ADCC Assay: Cytotoxicity of SKBR3 Tumor Cells

Fresh blood was drawn and diluted 1:1 with PBS containing 2% FBS. Lymphoprep (Sterncell Technologies) was added 1:2 to blood solution before centrifuging at 800 g for 20 mins. Serum, mononucleated cells, and lymphoprep were discarded and diluted BD Pharm Lyse 10x Buffer was added to RBC solution 1:10. After 15 min incubation, solution was centrifuged at 200 g for 5 minutes, decanted and washed in PBS with 1% FBS, and spun again at 200 g for 5 minutes. Pellet was resuspended in PBS with 2% FBS to a concentration of $5*10^7$ cells/ml. Fifty µL of Neutrophil enrichment cocktail (Stemcell Technologies), 100 µL magnetic nanoparticles and PBS 2% FBS were added to the suspension for a final volume of 5 mL. Neutrophils were isolated using a high power magnet from EasySep's Human Neutrophil Enrichment Kit. Cells were cultured in complete RPMI medium in the presence of 10 ng/mL G-SCF (PeproTech) and 50 ng/ml recombinant human IFN-γ (PeproTech) overnight. Antibodies were prepared at 40 ug/ml. Max lysis buffer was made from Triton X-100 at 2% v/v, SDS 1% w/v, 100 mM NaCl, and 1mM EDTA-2Na. In parallel, SkBR3 (Her2+ MDA-MB-453) cells were grown in McCoy's 5a complete media. Cells were passaged every 2-3 days until harvesting via Trypsin-EDTA. Pelleted cells were washed with PBS and resuspended in 1 ml ADCC culture media (RPMI 1640 with 10% ultralow IgG serum). Cells were labeled for 1 hr with Na51Cr04 (PerkinElmer Life Sciences). Labeled cells were washed in ADCC culture media and resuspended at desired concentration. Fifty µL tumor cells, 50 µL antibody solution at 20 µg/ml, 2 µg/ml, and 0.2 µg/ml concentrations, and 100 µL neutrophils were added to each desired well of U-bottom tissue culture plate. Effector cells and target cells were cocultured in complete media in an E:T ratio of 50:1 for 4 hrs at 37° C in 5% CO_2 in RPMI with 10% FBS medium. Plate was centrifuged at

2000 rpm for 10 mins and aliquots of supernatant were analyzed for chromium-51 levels in a Beckman LS 6500 scintillation counter.

4. Results and Discussion

4.1 Alanine Mutagenesis

Eleven different mutants were made with a single alanine substitution in each. Figure 6 below shows IgA (gray) in a binding complex with FcαRI (orange). Since wild type IgA has a homodimeric heavy chain structure, there are two binding interfaces present. Residues were selected for their proximity to the FcαRI amino acid chain Additionally, modeling with Rosetta

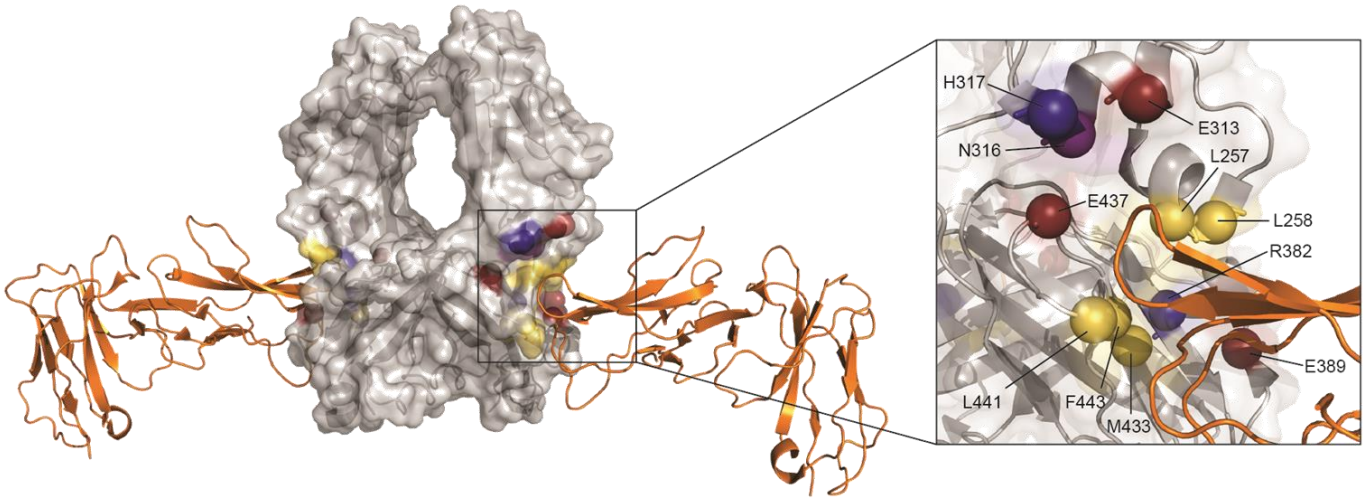


Figure 6: IgA residues selected for alanine substitution

software was used to resolve unclear parts of the crystal structure to help with residue selection (selected residues shown in Figure 6).

After selecting the residues, the mutants were cloned, expressed, and purified. Single amino acid substitutions often change protein expression, and assembly in addition to binding

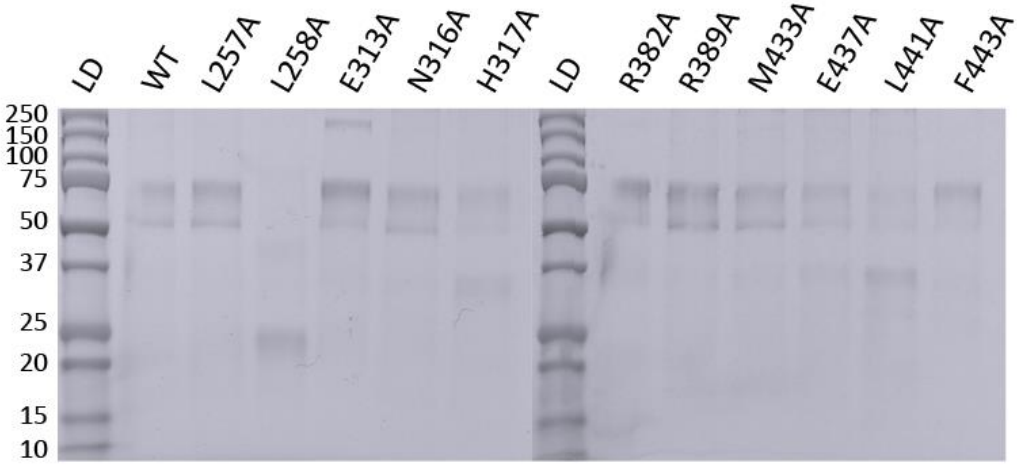


Figure 7: SDS-PAGE protein expression gel verifying mutant assembly

efficacy. Thus, two micrograms of each purified mutant protein was loaded in to a 4-20% polyacrylamide gel. The protein comprising each mutant was separated by molecular weight via gel electrophoresis (Figure 7).

The scale on left is in units of kilodaltons (kDa). An average IgA Fc domain has a molecular weight of about 25.2 kDa. A dimerized IgA Fc domain would have a molecular weight of 50.4 kDa. The brightest band for each lane should be around the 50 kDa mark. However, due to the addition of carbohydrate chains (glycosylation) some of the protein appears at a higher molecular weight. The heterogeneous mixture of added glycans slows down protein migration through the gel and account for the diffuse bands. Ten of the mutants show protein at the correct molecular weight with the exception of mutant L258A. Lack of displayed protein near the 50 kDa mark signifies the importance of this residue for assembly of the Fc dimer.

Finally, an ELISA was run to compare the relative binding affinities of all eleven mutants to FcαRI. IgA and IgG were included in the assay as a positive and negative control, respectively. Figure 8 shows the mutant binding affinities normalized to IgA. Significant binding residues display low affinity relative to wild type IgA because the alanine mutation changes binding capacity. The colored dots represent residue importance: green for low, orange for medium, and red for high. The question mark over L258A signifies the lack of correct protein

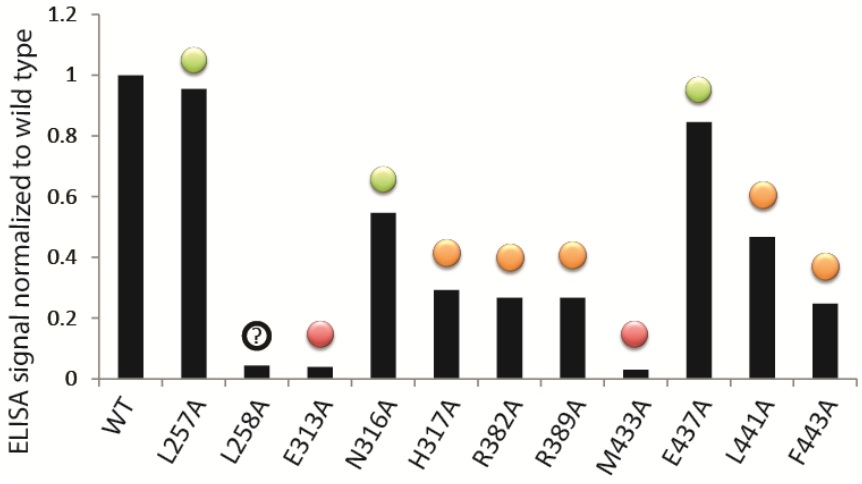


Figure 8: Relative affinities to FcαRI for 11 alanine substitution mutants

expression due to mutation of the 258th residue. These data show that the two residues marked with red dots, 313 and 433, are the most influential residues for binding to FcαRI. Since residue 433 is located in the IgA CH3 domain, we chose to insert this entire domain in to IgG.

4.2 Initial Mutant Binding Characterization

Mutants B, C, and D were constructed according to the schematic shown in Figure 1. Multiple ELISAs were performed to fully characterize our mutants with respect to an array of Fc receptors. These antibodies were made by substituting domains from IgA in to a backbone of IgG. The goal was to engineer an antibody that retains native IgG binding to the FcγRs but also has de novo binding to FcαRI, a receptor characteristic of IgA. Preliminary ELISA results are shown in figures 4 and 6 below.

Out of the three mutants, Mutants C and D showed the highest binding to FcαRI. Figure 9 shows the absorbance curve of the ELISA against an increasing dilution factor. Mutant D was chosen over Mutant C due to the SDS-PAGE protein expression gel shown in Figure 10. The single band of protein in lane D demonstrates better assembly of the mutant most likely due to the additional glycine residue. While mutant D did not show binding comparable to wild type IgA there is substantially higher binding than IgG, our negative control. More accurate quantification of binding affinity was further evaluated by SPR techniques.

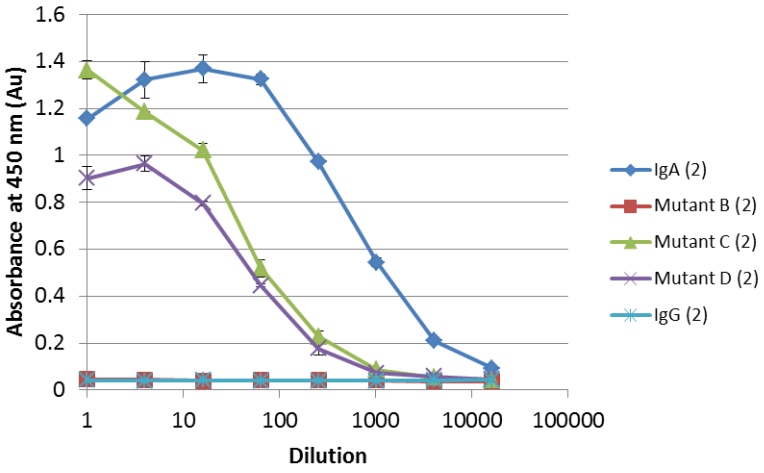


Figure 9: Mutant binding efficacy to FcαRI. Mutants C and D show highest levels of binding compared to IgA wild type.

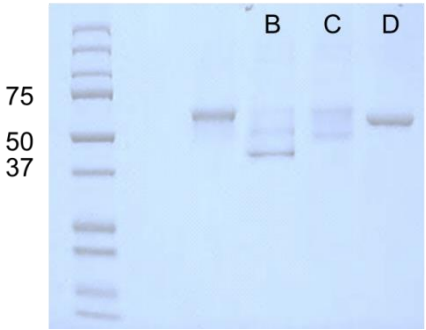


Figure 10: SDS-PAGE analysis of mutant Fc expression from HEK293F cells.

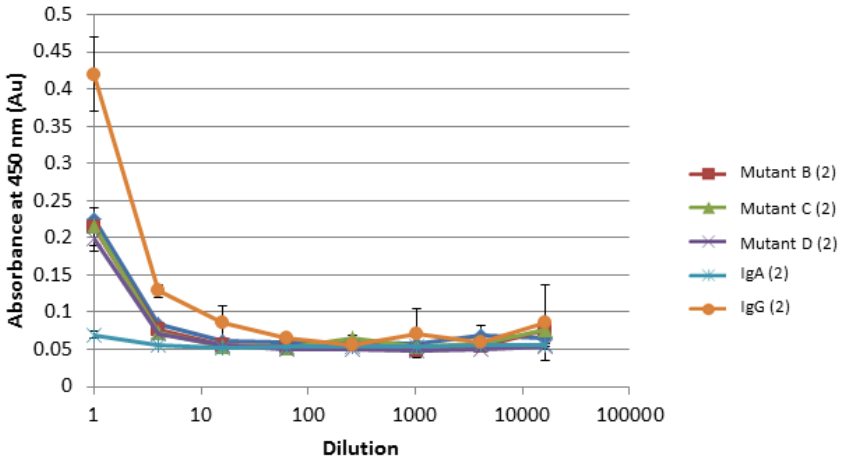


Figure 11: Mutant binding efficacy to FcγRI. Mutants B, C, and D all show close to equal binding retention.

Mutants B, C, and D all showed similar binding to FcγRI (Figure 11). As expected, our positive control of wild type IgG showed the most binding to FcγRI. Our engineered mutants appeared to have about half the level of FcγRI binding as our positive control. The negative control, wild type IgA, showed almost zero binding to FcγRI. These data indicate at least some retention of binding to the Fc gamma receptor class.

Due to the therapeutic importance of the Fcy receptors, ELISAs were run on the rest of the gamma receptor class to determine our mutants' binding efficacy. Our engineered mutants, in comparison to IgG, had significantly higher binding to FcγRIIA. And, perhaps more importantly, mutants B, C, and D all had very low binding to FcγRIIB. High binding efficacy to IIA and low binding efficacy to IIB is desired for effective antibody based therapeutics. A summary of our

Table 1: Summary of binding efficacies to important Fc receptors. Quantity of dots symbolize level of binding.

	FcαRI	FcγRI	FcγRIIa	FcγRIIb	FcγRIIIa
IgA	●●●	-	-	-	-
Mutant B	-	●●●	●	●	-
Mutant C	●●	●●●	●●●	●	-
Mutant D	●●	●●●	●●●	●	-
IgG	-	●●●●	●●●	●●●	●●●

ELISA data is displayed in Table 1. These results show that our engineered mutants have at least some of the desired characteristics of wildtype IgG and IgA antibodies. However binding to FcγRIIIA was almost non-existent. Any small perturbation in the CH2/CH3 domain structure seemed to negatively affect FcγRIIIa binding. To further explore FcγRIIIa sensitivity, Mutant A was designed and tested for CD16a efficacy.

4.3 Mutant A characterization

Mutant A only contains the CH2 binding loop and the additional glycine flexor without any CH3 residues from IgA. In contrast, Mutants B-D all contained domain swaps of the CH3 domain and also almost completely lost binding to FcγRIIIa. Mutant A was designed to test whether the CH3 swap was the main cause of CD16a binding loss. Results from the ELISA are shown in Figure 12. Mutant A and Mutant D show negligible binding to CD16a compared to the IgG positive control. FcγRIIIA binding must be very sensitive to conformational changes in the CH2 or CH3 domain. It is possible that IgG native residues in the CH2 binding loop and CH3 domain are necessary for CD16a binding. These data show that even small changes in the IgG CH2 domain will disrupt CD16a binding highlighting the difficulty of retaining binding to this receptor in the construction of a hybrid antibody.

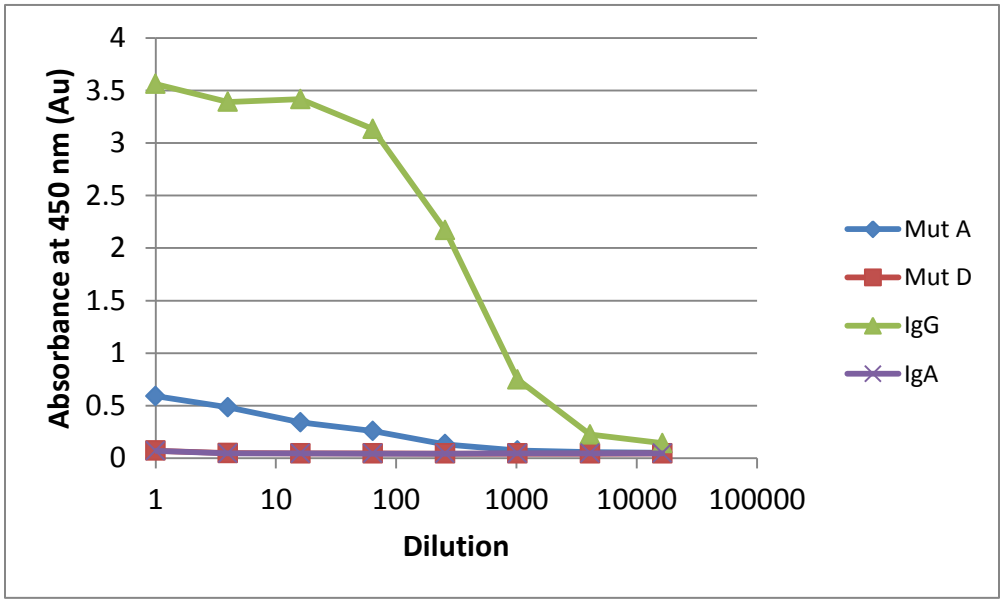


Figure 12: Binding efficacy to FcγRIIIA. Mut A and Mut D have significantly lower binding than the IgG positive control.

4.4 The Importance of IgA Glycosylation

In eukaryotic and prokaryotic cells, ribosomes are responsible for translating mRNA strands in to amino acid chains. Select proteins are modified further by the endoplasmic reticulum and golgi apparatus. These organelles often add carbohydrate chains, or glycans, to chosen functional groups of the protein. This type of post-translational modification known as glycosylation influences protein structure and function.

Producing consistent antibody glycosylation profiles among different expression batches is often very difficult. If a specific glycosylation pattern is needed to obtain correct antibody functionality, this antibody-based drug would be more expensive to produce and may not clear the FDA. In this experiment we analyzed the the importance of human IgA glycosylation to determine the extent of glycan matching that would be needed to commercialize our mutants. First, we expressed IgA in bacterial cells. The E. Coli used cannot glycosylate and as a result expressed IgA without any additional glycan attachments. Figure 12 indicates the assembly of IgA Fc in bacteria was not perfect due to the presence of a monomeric Fc fraction. Regardless, non-glycosylate3 IgA Fc was compared to glycosylated IgA Fc isolated from human serum by ELISA. IgA in human serum is primarily monomeric (about 85-90%) with a minor percentage in polymeric form.[29] An ELISA test was used to compare relative binding to FcαRI. Even with

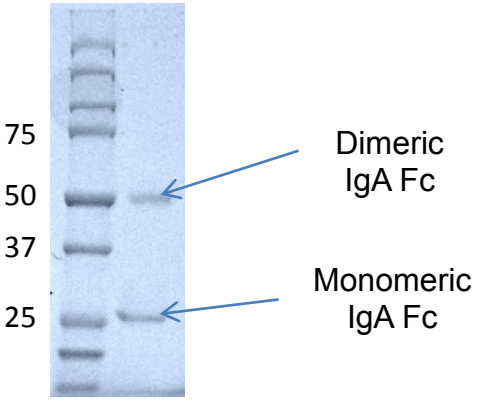


Figure 13: SDS-PAGE of purified IgA Fc expressed in bacteria

	FcαRI
Serum IgA	●●●
Bacterial IgA Fc	●●

Figure 14: Relative binding of Serum IgA Fc and Bacterial IgA Fc to FcαRI. Black dots indicate relative binding levels.

glycosylation and dimerization discrepancies, levels of FcαRI binding to human IgA Fcs and IgA Fcs expressed in bacteria were similar (Figure 14). From this experiment we concluded that glycosylation of IgA Fc is not critical for FcαRI binding. These results indicate that glycosylation and assembly differences between our HEK293 expression system and the natural human protein expression system are negligible. As a result, we can express aglycosylated IgA-derived mutants in bacteria without suffering a significant decrease from WT binding.

4.5 Surface Plasmon Resonance (Biacore)

Surface plasmon resonance tests using a Biacore 3000 machine were performed to verify ELISA results. This technique is used to examine the equilibrium dissociation constants between a protein (ligand) and its analyte, in this case between an Fc domain and its Fc receptor. Wild type IgA was used as a positive control and bovine serum albumin (BSA) was used to subtract non-specific receptor binding (negative control). Biacore tests are more robust and more quantitative than the simple ELISA tests described above. Binding between Mutant D and FcαRI, FcγRI, FcγRIIa, and FcγRIIb was examined to fully characterize our antibody mutant.

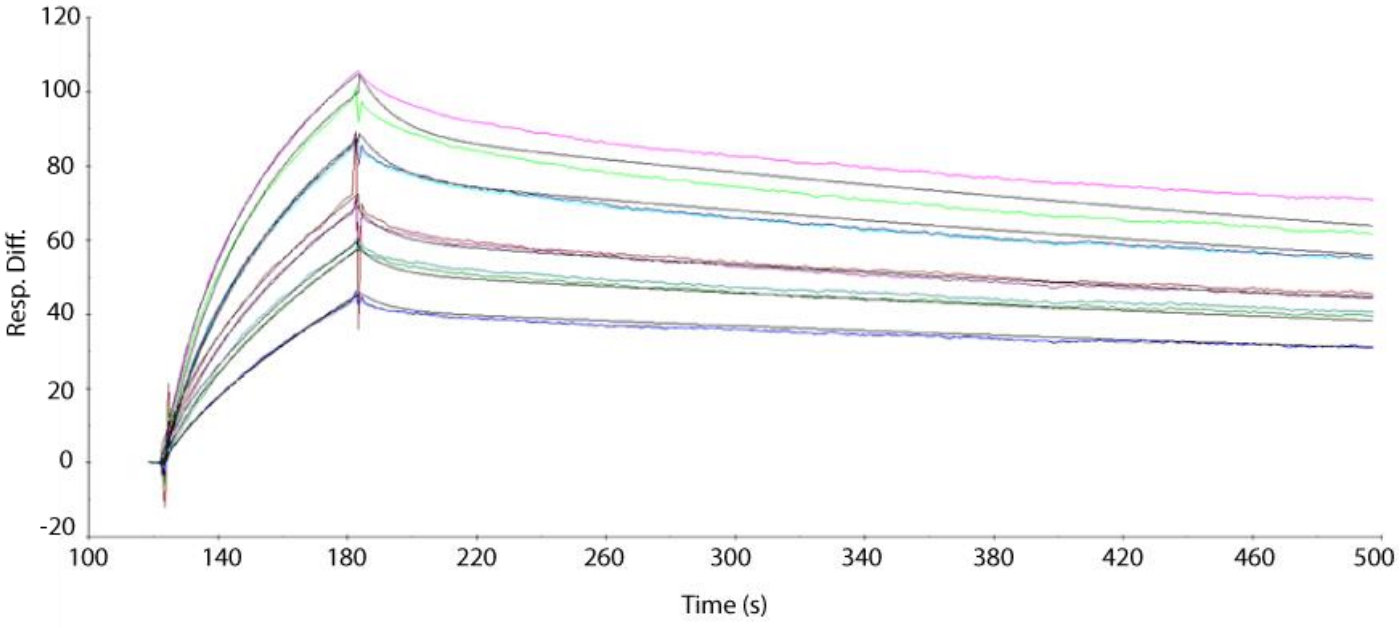


Figure 15: Biacore (SPR) curve fit for IgA complexed with FcαRI. Smooth association and gradual dissociation indicates high levels of binding. Each curve corresponds to specific injection concentration of IgA.

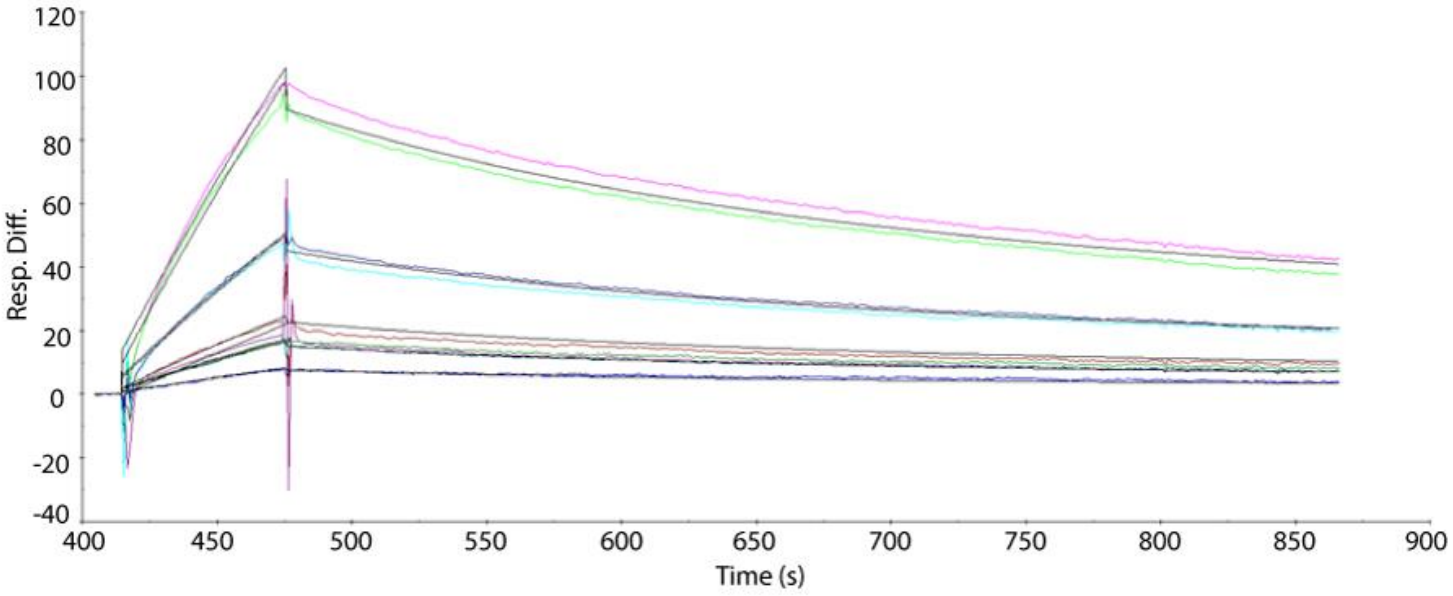


Figure 16: Biacore (SPR) curve for Mutant D complexed with FcαRI. Sharp increase and distinct drop-off indicates lower levels of binding. Each curve corresponds to specific injection

Curves returned by Biacore injections consist of refractive index values over time. The gradual increase in refractive index represents antibody-Fc receptor binding while the gradual decrease represents antibody-Fc receptor dissociation. These curves were fit to a monovalent Langmuir or bivalent analyte model to obtain association (k_a) and dissociation (k_d) constants. The Biacore curves for IgA complexed with FcαRI and Mutant D complexed with FcαRI are shown in figures

Table 2: Comparison of K_d and χ^2 values for IgA and Mutant D binding to different Fc receptors.

	FcαRI		FcγRI		FcγRIIa		FcγRIIb	
	μM	χ^2	nM	χ^2	μM	χ^2	μM	χ^2
IgA	0.76	1.07	-		-		-	
Mutant D	1.38	2.78	4.6	5.87	1.6	15	18	4.78

15 and 16, respectively.

A summary of the dissociation constant data obtained via SPR analysis is shown in Table 2. The dissociation constants are listed in the first column under each Fc receptor heading. The second column displays the chi-squared (χ^2) values for the Langmuir or bivalent analyte curve fitting. Chi-squared values represent the goodness-of-fit of a regression method in which a lower

χ^2 indicates a more exact fit. The χ^2 value for Mutant D binding to FcαRI is about double the χ^2 value for IgA binding to FcαRI. These data indicate that our engineered mutant D displays slightly more than half the original IgA binding to FcαRI binding. Chi-squared values for Mutant D binding to Fcγ receptors were compared to known IgG-Fcγ χ^2 values. It is important to note that we have low confidence about the K_d value for FcγRIIa due to the high χ^2 value.

A binding comparison between Mutant D and wild-type IgA/IgG is shown in Figure 17. Mutant D showed about 55% of de novo FcαRI binding while retaining about 75% of FcγRI and FcγRIIa binding. Surprisingly, mutant D also displayed very low levels of FcγRIIb binding. Since RIIb is an inhibitory protein, minimizing interaction with this Fc receptor is expected to increase potency of ADCC or ADCP in the body.[30] However, mutant D binding to FcγRIIIa is almost non-existent. Since FcγRIIIa is an important activating receptor, lack of Mutant D binding could decrease therapeutic potency, especially when NK cell activity is needed.

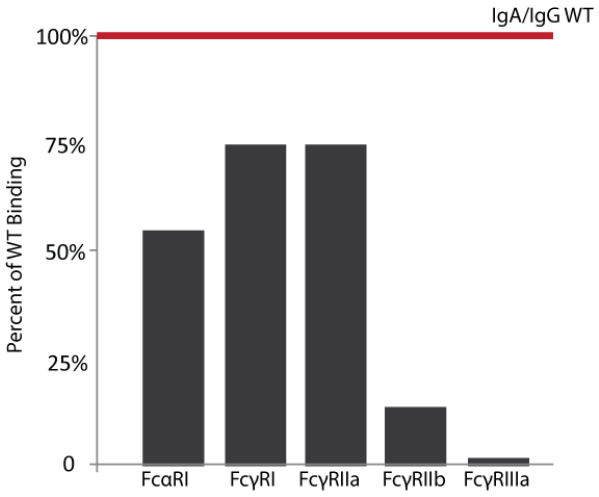


Figure 17: Binding comparison between mutant D and WT IgA/IgG. Gray bars indicate percentage of wild type binding displayed by mutant D.

4.6 ADCC Results

An ADCC assay was designed to test tumor cell cytotoxicity. An overview of the experiment is shown in figure 18. Pure lysis buffer was used as a positive control to release the maximum amount of chromium 51 isotope into solution.

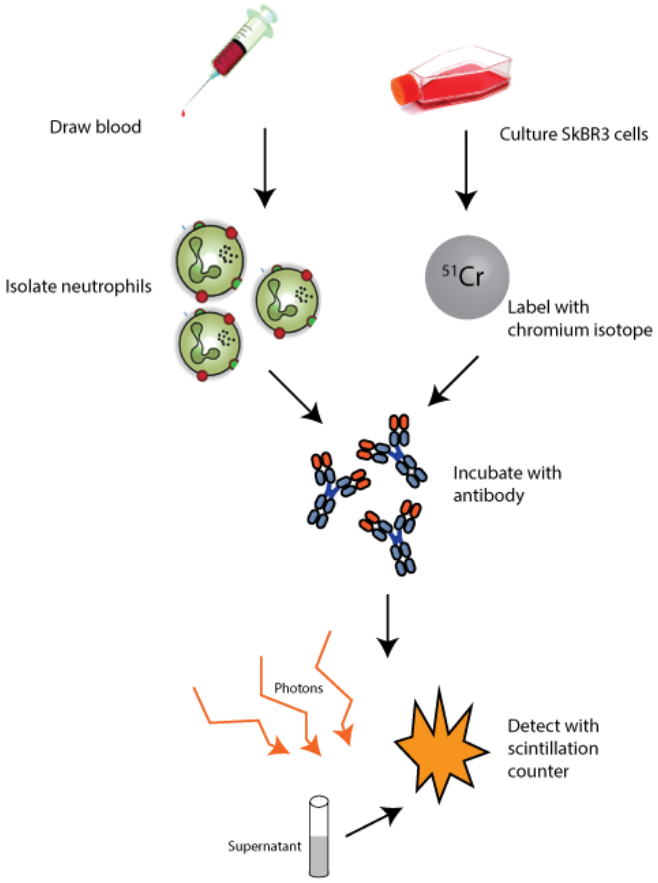


Figure 18: Overview of chromium-51 release assay to measure tumor cell cytotoxicity.

Additionally, a well of mixed neutrophils and tumor cells without antibody was used as a negative control to measure background levels of antibody independent chromium release. Since human neutrophils naturally express FcαRI at higher levels than FcγRI, G-CSF and human IFN-γ were used to increase FcγRI expression by mimicking a state of immune activation during overnight culture.[31] The results from the ADCC experiment are summarized in Figure 19. IgG

displayed surprisingly low levels of tumor cytotoxicity, possibly due to ineffective activation of FcγRI. The neutrophils may need to be activated longer for higher FcγRI expression. IgA levels of cytotoxicity were significantly higher than those of IgG which is expected due to the high expression of CD89 on neutrophils.[32] Mutant D showed higher cytotoxicity thanIgG but lower than IgA. Since mutant D binds to CD89 at slightly lower levels than IgA as shown from ELISA and SPR data, it is reasonable that mutant D is less cytotoxic than WT IgA. However, the data shows that partial activity through FcαRI makes mutant D more cytotoxic than IgG alone. Since IgG is the therapeutic standard for monoclonal antibody drugs, engineering an antibody with higher cytotoxic function than IgG is a significant step forward in the field of antibody immunotherapy.

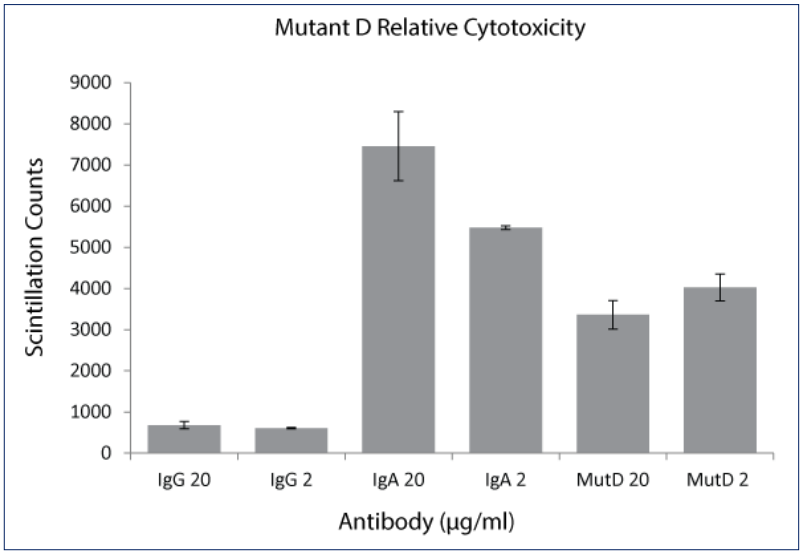


Figure 19: ADCC assay results showing relative levels of tumor cell killing from IgG, IgA, and Mutant D.

5. Conclusions

The idea of treating cancer with antibody-based therapies dates back to the 1960s when antigen expression was examined using serological techniques.[33] Since then, the field has evolved and only recently advanced to a stage necessary for drug creation. Since 1997, twelve antibodies have received approval by the FDA for treatment of solid tumor and blood-based malignancies.[34] All 12 of these antibody drugs are made from the IgG isotype. Since IgG is the most common antibody class in human blood, no approved drug uses any other antibody isotype. However, exclusivity of an antibody class results in limitations of immune cell interactions. Engineering an Fc domain that interacts with more, unique Fc receptors can overcome this class limitation.

IgA was chosen as a desired second class because of its interaction with FcαRI. This Fc receptor is expressed on cells of myeloid lineage including neutrophils. Since neutrophils are the most common type of white blood cell, their recruitment can lead to a formidable source of cytotoxic effector cells.[35] Additionally, it has been demonstrated that FcαRI is the most effective neutrophil Fc receptor to induce antibody-dependent tumor cell killing, even more so than FcγRI.[18] Creating an antibody that engages both FcαRI and Fcγ receptors could result in even more potent tumor cell killing through the action of different immune cells.

After examining binding complexes on PyMOL and verifying residue importance through alanine scanning, we constructed four mutants that consisted of IgA domains grafted in to IgG. Instead of manipulating individual residues, we decided to swap large domains or loops to bolster expression, increase our chances of de novo binding, and decrease immunogenicity. Mutant D turned out to have the best combination of binding to FcαRI and protein expression. Quantification of Mutant D binding through SPR helped to fully characterize our antibody. Mutant D exhibits about 55% of IgA binding to FcαRI and retains about 75% of IgG binding to

FcγRI and FcγRIIa. Additionally, our engineered antibody does not bind very well to FcγRIIb (~12%) or FcγRIIIa (~2%). Retention of some Fcγ receptor binding along with new binding to FcαRI confirms our belief that hybrid binding is possible.

Results from the SkBR3 ADCC assay verify that the introduction of new FcαRI receptor binding enhances tumor cell killing. Mutant D exhibits higher tumor cell cytotoxicity than IgG alone. The impact of this discovery could be very significant. As a platform for countless tumor specific variable domains, our engineered Fc could serve as an integral part of many future monoclonal antibody drugs. IgA may start to be used more as a therapeutic agent. In the future, hybrid Fc domains that engage many different immune cells may become commonplace in the field of cancer immunotherapy.

6. Future Work

In order to increase the impact of our discovery, a few more experiments must be run. First, we must examine FcγRI expression levels on neutrophils. IgG exhibited surprisingly low levels of cytotoxicity which may be a result of a lack of Fcγ receptors on the experimental neutrophils. Although G-CSF and IFN-γ were supposed to stimulate FcγRI expression on the neutrophil surface, the levels of FcγRI expression should be confirmed by FACS. The ADCC assay must then be repeated to verify our findings.

A different cell-based assay must be performed to show that Mutant D has higher cytotoxicity than IgA in certain circumstances. In the presence of neutrophils, wild type IgA will most likely be preferred. However, in the presence of other immune cells that mostly express Fcγ receptors, mutant D may be preferred over IgA. Our results would have a higher impact if we should mutant D preference over WT IgA. In the future we may test the cytotoxicity of Mutant D in the presence of dendritic cells and/or full PBC fraction.

After confirming the *in vitro* cytotoxicity of our antibody, we plan on testing our antibody in an immune compromised cancerous mouse. We will adoptively transfer human immune cells to the mouse along with our antibody to measure to measure *in vivo* effectiveness. Tumor cell count can be measured over time after the administration of our antibody. Comparing mutant D tumor cell killing to wild type IgA and IgG can corroborate our *in vitro* data and further increase the importance of our engineered mutant.

7. Acknowledgements

As with most of scientific research, my work has been collaboration among many different Georgiou lab members. First, I would like to thank Dr. George Georgiou, my first reader and the lab's principal investigator. He has introduced me to the exciting field of protein engineering and given me a chance to work on great, high impact research. Dr. Haley Tucker, my second reader, has always been encouraging and helped jumpstart my research career by taking me in to her lab as a freshman. I also owe a great deal of thanks to William Kelton, the graduate student who has mentored and coached me ever since I joined the BIGG lab. He has allowed me to work on his projects and has always been extremely helpful along the way. Special thanks to Chhaya Das, Jiwon Lee, Tae Hyun Kang, Dr. Oana Lungu, and Dr. Takaaki Kojima for helping out with experiments and data collection. Finally, I would like to thank the rest of the Georgiou-Iverson lab for being very helpful and creating a fun, productive place to work.

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9. Author

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